Probabilistic (Bayesian) Modeling of Gene Expression in Transplant Glomerulopathy

Eric A. Elster, MD FACS[1,2,3,4], Jason S. Hawksworth, MD[1,2], Orlena Cheng, PhD[3], David B. Leeser, MD FACS[2], Michael Ring, PhD[3], Douglas K. Tadaki, PhD[1], David E. Kleiner[5], John S. Eberhardt III, BA[6], Trevor S. Brown, PhD[1] and Roslyn B. Mannon, MD[3]

[1]Regenerative Medicine Department, Naval Medical Research Center

[2]Organ Transplant Service, Walter Reed Army Medical Center

[3] National Institutes of Diabetes and Digestive and Kidney Diseases, National Institutes of Health

[4]Department of Surgery, National Naval Medical Center

[5]Laboratory of Pathology, National Cancer Institute

[6]Decision Q Corporation

Corresponding Author: Eric A. Elster, M.D.
Regenerative Medicine Department
Combat Casualty Care
Naval Medical Research Center
Silver Spring, MD 20910
Email Address: eric.elster@med.navy.mil

Conception and design: Elster, Brown, Hawksworth, Mannon
Acquisition of data: Cheng, Ring, Hawksworth, Mannon
Analysis and interpretation of data: Hawksworth, Eberhardt, Brown, Elster, Mannon
Drafting of manuscript: Hawksworth, Elster, Brown, Mannon
Critical revision: Elster, Mannon, Tadaki
Statistical expertise: Eberhardt, Hawksworth, Brown, Mannon
Obtaining funding: Elster, Tadaki, Mannon
Supervision: Elster, Mannon

 This effort was supported (in part) by the U.S. Navy Bureau of Medicine and Surgery and the National Institutes of Health.

Running Title: **Bayesian Modeling of TG Gene Expression**

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2010		**00-00-2010 to 00-00-2010**

4. TITLE AND SUBTITLE
Probabilistic (Bayesian) Modeling of Gene Expression in Transplant Glomerulopathy

5a. CONTRACT NUMBER
5b. GRANT NUMBER
5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

5d. PROJECT NUMBER
5e. TASK NUMBER
5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Naval Medical Research Center,Regenerative Medicine Department,Combat Casualty Care,Silver Spring,MD,20910

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)
11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES
Journal of Molecular Diagnostics 2010, Vol. 12, No. 5

14. ABSTRACT

Transplant glomerulopathy (TG) is associated with rapid decline in glomerular filtration rate and poor outcome. We utilized low-density arrays with a novel probabilistic analysis to characterize relationships between gene transcripts and the development of TG in allograft recipients. Retrospective review identified TG in 10.8% of 963 core biopsies from 166 patients; patients with stable function (SF) were studied for comparison. The biopsies were analyzed for expression of 87 genes related to immune function and fibrosis using real-time PCR, and a Bayesian model was generated and validated to predict histopathology based on gene expression. A total of 57 individual genes were increased in TG compared with SF biopsies ($p<0.05$). The Bayesian analysis identified critical relationships between ICAM-1, IL- 10, CCL3, CD86, VCAM-1, MMP-9, MMP-7, and LAMC2 and allograft pathology. Moreover, Bayesian models predicted TG when derived from either immune function {AUC (95% CI) of 0.875 (0.675-0.999) p=0.004} or fibrosis {AUC (95% CI) of 0.859 (0.754-0.963), $p<0.001$} gene networks. Critical pathways in the Bayesian models were also analyzed using the Fisher exact test and had p-values < 0.005. This study demonstrates that evaluating quantitative gene expression profiles with Bayesian modeling can identify significant transcriptional associations that have the potential to support the diagnostic capability of allograft histology. This integrated approach has broad implications in the field of transplant diagnostics.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**30**	

ABSTRACT

Transplant glomerulopathy (TG) is associated with rapid decline in glomerular filtration rate and poor outcome. We utilized low-density arrays with a novel probabilistic analysis to characterize relationships between gene transcripts and the development of TG in allograft recipients. Retrospective review identified TG in 10.8% of 963 core biopsies from 166 patients; patients with stable function (SF) were studied for comparison. The biopsies were analyzed for expression of 87 genes related to immune function and fibrosis using real-time PCR, and a Bayesian model was generated and validated to predict histopathology based on gene expression. A total of 57 individual genes were increased in TG compared with SF biopsies ($p<0.05$). The Bayesian analysis identified critical relationships between ICAM-1, IL-10, CCL3, CD86, VCAM-1, MMP-9, MMP-7, and LAMC2 and allograft pathology. Moreover, Bayesian models predicted TG when derived from either immune function {AUC (95% CI) of 0.875 (0.675-0.999), $p=0.004$} or fibrosis {AUC (95% CI) of 0.859 (0.754-0.963), $p<0.001$} gene networks. Critical pathways in the Bayesian models were also analyzed using the Fisher exact test and had p-values < 0.005. This study demonstrates that evaluating quantitative gene expression profiles with Bayesian modeling can identify significant transcriptional associations that have the potential to support the diagnostic capability of allograft histology. This integrated approach has broad implications in the field of transplant diagnostics.

INTRODUCTION

Long-term kidney allograft function continues to improve only modestly, despite dramatic improvements in acute rejection rates and short term patient and graft survivals.[1]In spite of its limitations, measurement of serum creatinine remains the primary monitoring modality following kidney transplantation. Significant changes in serum creatinine, and/or the development of proteinuria, result in a series of maneuvers to define the many potential etiologies of acute and chronic allograft dysfunction. Allograft biopsy is the "gold-standard" of these maneuvers, although morphologic analysis may not easily distinguish these etiologies. Furthermore, the analysis may be limited in regards to prognostic importance and functional outcome. Thus, identification of biomarkers of allograft failure and the development of tools for their interpretation is of critical interest, both in providing disease detection in a more sensitive and specific fashion, and in allowing sufficient lead time for intervention. Additionally, such markers may allow for risk assessment and medical-regimen tailoring that is personalized to provide optimum outcomes.

Transplant glomerulopathy (TG) is a disease of the kidney allograft initiated by endothelial injury. Morphologically, there is widening of the subendothelial space with accumulation of debris, mesangial interpositioning, and matrix deposition in the glomerular capillary wall, as well as capillary wall double-contouring in the absence of immune complex deposition.[2] Electron microscopy may show endothelial cell separation from the glomerular basement membrane prior to light microscopic changes. The etiology of TG is under considerable scrutiny. Prior studies implicated an antibody mediated response[3-5], but this has not been consistently demonstrated.[6, 7] Accompanying this lesion may be evidence of chronic injury, including interstitial fibrosis and tubular atrophy (IF/TA), the hallmarks of chronic allograft nephropathy.[8] Clinical presentation often occurs a year or more after transplantation, although in the context of protocol kidney biopsies, light microscopic changes may be seen earlier, with associated proteinuria, hypertension, and a progressive decline in function culminating in graft loss.[9] Importantly, there is no specific effective

therapeutic strategy beyond augmentation of immunosuppression. Thus, identifying pathogenic mediators not only for therapeutic purposes but also for early identification may lead to improved outcomes.

In this study, we assess the potential of a novel diagnostic method utilizing custom low density gene expression arrays and machine learning algorithms in an effort to determine the transcriptional features associated with TG and to begin to identify biomarkers that may be indicative of TG. While there has been some research in identifying biomarkers of TG, we have yet to see the evaluation of a systems biology approach to this problem. We focused on transcripts that have been associated with other forms of acute and chronic renal allograft injury in kidney allograft recipients with the intent of evaluating a systems biology modeling approach. Initial data analysis using conventional statistical methods confirmed the proinflammatory state of this lesion.[10] Incorporation of these data utilizing machine-learning software, however, derived statistically significant yet substantially novel associations between individual transcripts. We performed this analysis specifically to assess the potential value of a graphical, hierarchical model of conditional dependence in generating novel hypotheses and providing guidance in patient classification. Moreover, the resulting model provides insight into the probable pathogenesis of TG and a set of potential biomarkers to test and characterize recipients at risk for disease. These results highlight the hypothesis-generating potential of this method by elucidating potential pathways for investigation and the decision-supportive utility of defined, quantitative classification models of disease versus health states.

METHODS

Patient selection and evaluation

Protocols were approved by the Institutional Review Board of the National Institutes of Health and included informed consent. Retrospective review of 963 renal transplant core biopsies (166 patients) identified TG in 20 biopsies (18 patients; 10.8%). A cohort of 32 biopsies (19 patients) of stable function

(SF) allografts was studied for comparison. SF was defined as at least 6 months post-transplant without change in renal function and the absence of any significant histological or clinical abnormalities.

Immunosuppression included induction in 94.6% (n=35) using rabbit anti-thymocyte globulin (40.5%; n=15), Alemtuzumab (29.7%; n=11), Daclizumab (18.9%; n=7), or solumedrol alone (5.4%; n=2). Patients were maintained on monotherapy with tacrolimus or sirolimus (56.8%; n=21), triple immunosuppressive therapy including tacrolimus or sirolimus, MMF, and prednisone (29.7%; n=11), or other maintenance regimen variations (14.5%; n=5).

Patients were routinely screened post-transplant for anti-HLA antibodies with solid phase Class I and II ELISA or multi-antigen synthetic flow bead (Tepnel, Stamford, CT) testing with the Luminex system (Luminex Inc., Austin, TX). Positive sera were subsequently tested using specific HLA antigen-coated flow beads (One Lambda, Inc., Canoga Park, CA).

Biopsy acquisition, preparation, and evaluation

Protocol biopsies were obtained routinely at time of transplantation and at 1, 6, 12, 36, and in some cases 60 months. A portion of the cortex was snap frozen as previously described.[11] The percent cortex was obtained for each sample for validation. For all biopsies this was 75.71±25.52. The remaining portions were fixed in formalin, sectioned, and stained.

All biopsies were evaluated in masked fashion by a single dedicated pathologist and scored using the Banff classification.[12-14] The diagnosis of TG was based on the presence of glomerular basement membrane duplication and severity scored on the percentage of glomerular peripheral capillary loops involved in affected, non-sclerotic glomeruli.[13] Patients with duplication of the glomerular basement membrane due to recurrent disease were excluded. C4d staining was performed in 43/52 samples using an immunoperoxidase technique.

Quantitative real-time polymerase chain reaction (qPCR)

RNA was extracted and converted to cDNA.[15] cDNA (100ng) was used for qPCR using a Low Density Array (Applied Biosystems Inc., Foster City, CA).[16] Two groups of targets (Table 1) were quantitated: 43 transcripts related to general immune function (Gene Panel 1 – GP1) and 45 transcripts related to allograft fibrosis pathways (Gene Panel 2 – GP2). Each target was analyzed in quadruplicate. 18S ribosomal RNA was used as an internal control. Individual samples were compared with pooled cDNA from live donors undergoing open donor nephrectomy. Transcript quantification was derived using the comparative threshold cycle method,[16] and reported as *n*-fold difference.

Statistical Analysis

SF and TG patient clinical variables were compared and analyzed using SPSS (SPSS 16.0, SPSS Inc., Chicago, IL). Associations between categorical variables were studied with Fisher exact test or χ^2test, as appropriate. Continuous variables of normally distributed data were assessed with the Student *t*-test. Relative-fold expression data were compared using independent sample Student *t*-test. A two-tailed p value < 0.05 was considered statistically significant.

Multivariate dependence relationships were also analyzed with FasterAnalytics™ modeling software (Decision Q, Washington, DC), a machine learning software package used to develop graphical models of conditional dependence (Bayesian networks). Machine learned Bayesian networks identify conditional dependence between variables and present this structure to the user in an intuitive, graphical format. Preliminary modeling included diagnosis (DX; TG or SF), C4d staining grade (Banff C4d; 0, 1, 2, or 3), and each gene of either GP1 or GP2. Quantitative transcript relative-expression data were categorized by distribution into three equal probability density groups. Models were derived in step-wise iterations until the optimal network was identified as determined by cross-validation and qualitative assessment against clinical experience and the literature. To evaluate robustness of the Bayesian models, 10% of the biopsy

data were randomly excluded from each dataset in 10 non-overlapping iterations for a 10-fold cross-validation.[17] To further validate the statistical significance of our findings, we used the directed graph produced by the model and performed a Fisher's exact test to quantify the statistical significance of each arc on a bi-variate basis between the nodes at each end of the arc. Series divided into the same reference ranges as in the Bayesian network and for each pair, a p-value was calculated using Fisher's exact test.

RESULTS

Patient demographics and biopsy characteristics

Patient demographics were matched between the SF and TG patients (Table 2). Patients with TG had higher mean pre-transplant peak PRA and were more likely to have had a prior transplant, but these associations were not statistically significant. However, patients with TG had a significantly higher incidence of post-transplant donor specific antibody (DSA) and graft loss, $p<0.05$, consistent with prior reported experiences.[5, 8]

Mean creatinine at time of biopsy was significantly higher in TG (2.5 ±1.1 mg/dL) compared with SF (1.3 ±0.3 mg/dL) allografts ($p<0.001$). Significantly more biopsies from TG allografts were obtained for clinical cause ($p=0.001$; Table 3), and, not surprisingly, C4d immunostaining was more often positive in TG biopsies than in SF ($p=0.013$). By definition, the TG biopsies had a higher chronic glomerulopathy (CG) grade (2.7 ±0.5) than SF biopsies, $p<0.001$.

Allograft transcriptional profile

Thirty-two transcripts related to immune function were significantly ($p<0.05$) increased in TG compared with SF, suggesting upregulation of inflammatory pathways involving multiple immune mechanisms (Figure 1A-G). Moreover, twenty-five transcripts related to interstitial fibrosis were increased in TG compared with SF ($p<0.05$).

Graphical Bayesian network of conditional dependence in transcript expression

Machine-learned Bayesian network modeling was used to identify conditional dependence relationships between gene transcript expression data and allograft pathology (TG versus SF as defined in Methods). GP1 and GP2 expression data were modeled independently and optimized relative to diagnosis. Below we focus on those genes found to be most interdependent on diagnosis within the generated networks.

In GP1 (Figure 2A), ICAM-1, IL-10, CCL3, and CD86 shared the strongest conditional dependence with diagnosis in the machine-learned model. To further validate our findings, we used the associations identified in the GP1 Bayesian network to perform Fisher exact testing and found the conditional dependence associations to be highly statistically significant, with all associations having a p-value of 0.01 or less. (Table 4) Similar to our findings above, multiple additional T-cell function, costimulatory, chemotaxis, and cytokine transcripts were also related to allograft pathology. In SF, relative transcript expression is lower within the network (Figure 2B); conversely, in TG the relative expression of these transcripts is generally higher than in the controls (Figure 2C). In this, the unsupervised, machine-learned Bayesian analysis has identified intriguing novel and potentially important relationships among the gene expression profiles and allograft pathology for further exploration and biological validation.

This Bayesian network further enabled investigation of the possible influence of specific, coincident gene expression on allograft pathology. More specifically, classification model GP1 allowed us to estimate the probability of outcome in the study population by using available evidence and without necessitating a full understanding of the underlying biological pathways. Setting the evidence of coincidentally elevated expression of ICAM-1 (≥1.84 fold), IL-10 (≥16.9 fold), and CCL3 (≥3.15 fold) increased the probability of TG to 99.67% as opposed to SF (Figure 3A). This served as a demonstration of allograft-outcome probability calculation using transplant-specific gene expression data and an internally cross-validated model.

With increased expression (>8.89 fold) of the costimulatory molecule CD86, multiple related transcripts are also increased as illustrated by the adjusted probability distributions throughout the network (Figure 3B). In addition, by increasing only CD86 expression, the probability of TG also increased to 80.61%. This interactive, evidence-based feature of Bayesian network analysis facilitates elucidation of potentially novel biological pathways.

Comparative transcript and histological Bayesian network probability of allograft pathology

In the Bayesian model of GP2, VCAM-1, MMP-9, MMP-7, and LAMC2 are critically related to pathology (Figure 4A). To further validate our findings, we used the associations identified in the GP2 Bayesian network to perform Fisher exact testing and found the conditional dependence associations to be highly statistically significant, with all associations having a p-value of 0.001 or less. (Table 5) Coupling histological criteria with transcript expression in the context of a cross-validated classification model provided a powerful predictor of allograft pathology. The probability of TG with a C4d grade of 3 alone is 81.25% (Figure 4B; 4D) while, not unexpectedly, the probability of SF with a C4d grade of 0 is 83.9% (Figure 4D). However, when coupled to increased expression of VCAM-1 (≥1.96 fold), MMP-9 (≥5.34 fold), MMP-7 (≥2.77 fold), and LAMC2 (>2.19 fold) the probability of TG increased to 99.67% (Figure 4C). Furthermore, with increased expression of only LAMC2 (>2.19 fold) and MMP-7 (>2.77 fold), the probability of TG is 95.6%; with decreased expression of LAMC2 (<0.52 fold) and MMP-7 (<1.04 fold), the probability of SF is 99.1% (Figure 4E).

Bayesian model validation for transcript network prediction of allograft pathology

Using 10-fold cross-validation analysis, both models for GP1 and GP2 estimated allograft pathology. GP1 estimated TG with an AUC (95% CI) of 0.875 (0.675-0.999), p=0.004 and sensitivity, specificity, positive predictive value, and negative predictive value of 85.7%, 87.5%, 92.3%,and 77.8%, respectively; GP2

estimated TG with an AUC (95% CI) of 0.859 (0.754-0.963), p<0.001and sensitivity, specificity, positive predictive value, and negative predictive value of 80.0%, 84.4%, 76.2%, and 87.1%, respectively.

Of interest is the models ability to predict outcome in sequential biopsies. Thirteen cross-validation models were generated using the same discretization as the overall Bayesian network (Table 6). For each training dataset, a single patient's multiple biopsies were removed. The removed patient data were then used to test the new validation model. This cohort of patients with multiple biopsies included 28 biopsies from 13 patients, of which 4 biopsies (2 patients) were diagnosed with TG and 24 biopsies were diagnosed as SF. A comparison of resulting model predictions to pathological findings yields one patient with one biopsy discrepantly identified, while the other biopsy had confirmatory identification; one patient with both biopsies discrepantly identified; two patients with both of their biopsies confirmed as TG; and nine patients with each of their biopsies confirmed as SF.

The analysis of sequential renal graft biopsies yielded consistent transcript expression profiles within GP2. Each biopsy, when classified by the same Bayesian network, exhibited probability of diagnosis similar to its subsequent, matched sample with the exception of two biopsies from one graft, patient 002-003. An additional point is that two of the three SF biopsies that were discrepantly classified as TG by the model were from the same graft, patient 002-014, and were in agreement with each other with a probability greater than 0.84.

DISCUSSION

In this study we attempted to characterize a panel of genes associated with TG using a novel machine-learning methodology producing Bayesian networks to evaluate a systems biology approach in identifying novel biomarkers of TG. As such, we have demonstrated that multiple gene transcripts associated with immune function and fibrosis are transcriptionally active in TG, have statistically significant association with outcome, and may have the potential to be highly predictive of abnormal

outcome. We believe that with further development and prospective clinical validation, this integrated approach will enable further understanding of this disease process and allow for the development of a clinically relevant diagnostic of allograft pathology and ultimately provide biomarkers for use in clinical trials.

When examined using traditional statistics, gene transcripts related to T-cell activation, effector function, costimulation, chemotaxis, and endothelial activation (Gene Panel 1) are all up-regulated in our recipients with TG (Figure 1). Furthermore, transcripts associated with epithelial-mesenchymal transformation, cytoskeleton structure, and growth regulation (Gene Panel 2) are also up-regulated. While it is evident that the transcriptional profile within biopsies histologically identified as positive for TG is markedly different from a stable functioning allograft, these differences indicate greater general immune activation without identifying specific pathways. Such findings have not been previously reported as many studies of TG have focused on histologic changes and association with alloantibody[18]. As the comprehensive interpretation of such datasets has remained difficult, we believe that our novel approach provides a method for the generation of new hypothetical pathways.

Methods to analyze complex, heterogeneous data sets incorporating the relationships between clinical, histological, and transcriptional variables have enormous utility in clinical research and application. In order to address the inherent analytical complexity of biomarker datasets, we have utilized machine-learned Bayesian network analysis. Bayesian theory relates the conditional independence of known events in order to compute posterior, or unknown, probabilities.[19, 20] Here, we have applied measured transcriptional data in order to determine the posterior probability of allograft pathology. While there are many analytical technologies available, we have focused on machine learning because of its inherent ability to address high-dimensionality multivariate data. We selected Bayesian networks, specifically, because the graphical models produced are transparent and intuitive, which allows the researcher to more

readily identify underlying mechanisms. This approach enhances many classical statistical analyses and has been employed for diagnostic and prognostic applications in oncology[21, 22] and cardiology.[23, 24]

When analyzed using Bayesian analysis alone, several conditional relationships become apparent within the transcript profiles. Specifically, ICAM-1, IL-10, CCL3, and CD86 are all conditionally related to outcome when modeling gene expression related to immune function (GP1). Additionally, VCAM-1, MMP-9, MMP-7, and LAMC2 are conditionally related to allograft pathology when analyzed with respect to fibrosis (GP2). As TG is rarely an isolated pathologic lesion,[8] the association of IL-10, CCL3, and CD86, although not histologically evident, may represent transcriptional overlay of cell-mediated inflammatory changes within the allograft.[25] Endothelial activation has been proposed as a mechanism for the pathogenesis of TG,[9] and interestingly, the machine-learned Bayesian network, GP1, associated two endothelial adhesion molecules, ICAM-1 and VCAM-1, directly to allograft pathology. TG is also morphologically characterized by duplication of the basement membrane, and appropriately, laminin (LAMC2), a major component of basement membrane, was also identified as a vital transcriptional indicator.

While many of these associations uncovered by the Bayesian model are novel, several well-established relationships support that such an approach has biologic relevance. The relationship between pathology and cell signaling (chemokine expression), cell trafficking (adhesion molecule expression) and tissue remodeling (MMP expression) as demonstrated by this analysis is supported by current models of TG. [26-29] [30] [9] TG is believed to be secondary to binding of donor specific antibodies to endothelium with resulting stimulation and recruiting of secondary mediators leading to an inflammatory response. [9] [30]This inflammatory response and subsequent tissue injury has been associated with chemokine, adhesion molecule and MMP expression. [26-28] [14, 29, 31] Additionally, adhesion molecule expression has been shown to be associated with both chronic disease and stable function in renal transplant recipients. [32] As demonstrated in our model, alteration of chemokine expression has been linked to costimulatatory

molecules (CD28,40L,80,86) and IL-10 has been demonstrated to be elevated in allografts with stable function. [26, 33 34-36] Finally, the development of TG and Cd4 expression has also been well characterized. [37 38 9] Thus, our preliminary data suggest that machine-learned Bayesian models may elucidate critical pathways related to the allograft pathology within transcriptional datasets.

While transcriptional analyses of renal transplant biopsies have been extensively reported, [11, 39, 40] this is the first report of an integrated panel with a graphical tool to define conditional relationships and potentially assist the clinician in prediction of transplant-specific diagnosis. As such, the ability to analyze several biomarkers at once offers several advantages over individual endpoints. This allows for the consideration of temporal changes individual biomarker levels vary with time during the disease process.[41] Further, it allows for the capture of feedback loops and inter-biomarker dependencies to improve sensitivity and specificity. Efforts in oncology and HIV have already introduced personalized medicine into clinical reality.[42] In order for a biomarker, or panel of biomarkers, to reach clinical utility as part of a personalized medicine approach, it would ideally meet the following criteria: inexpensive, easy and rapid quantification, detectable early in the course of the disease process, repeatable, and have a high degree of sensitivity and specificity.[43, 44] Based on our preliminary data, we believe that our panel of biomarkers, once prospectively clinically validated, may address these goals for the diagnosis of transplant pathology. PCR based assays, such as used herein, offer the potential of cost effectiveness, repeatability, and rapidity.[45] Additionally, given the relatively long-term survival of renal allografts, prospective application of such models to transplant biopsies may allow for early diagnosis, therapy alterations, and guidance in clinical trials.[2, 46]

Admittedly, this study is limited by the current dataset size and disease process. The dataset employed included patients with several different immunosuppressant approaches. While this reduces uniformity, we feel that it enhanced model robustness and favored broad utilization as the strategies used represent current standard practices. TG pathology is evolving, so there are limited treatment options. This limits

the ability to judge the effectiveness of a biomarker panel in addressing a clinical disease process; however, the ability to predict SF as described does offer the ability to affect therapeutic decisions. Patients quantitatively classified by the network as SF, as opposed to ongoing pathology, may benefit from immunosuppression weaning rather than continuous therapy, which has inherent toxicity, infection, and malignancy risks. Most importantly, the retrospective nature of this study does not allow for robust validation of the predictive models developed from the relationships derived from the transcriptional datasets. This will require additional prospective analysis with models such as described. Additionally, the small number of cases available for analysis renders the disease specific conclusions not definitive and requires further analysis. This is due to a lack of processed biopsies prior to the identification of TG via histologic findings. Therefore, given that many of the relationships uncovered by the model appear to be supported by other investigators, we believe that future analysis would be able to validate the current model and develop similar models for disease states where know therapeutic options exist, such as acute or antibody mediated rejection

In renal transplant recipients, we have demonstrated the ability to apply machine-learned Bayesian analysis of allograft biopsy gene expression to establish models of interdependent relationships and pathologic probabilities. The machine learning approach, which highlighted relationships not readily apparent with other analytical methods, provides a overview for biomarker analysis of stable function allograft as opposed to one exhibiting transplant glomerulopathy. We plan to continue this work in an effort to validate our biomarker panel for use as surrogate endpoints of clinical trials and clinical decision-making. As such, this effort signifies the introduction of personalized medicine in the realm of organ transplantation.

ACKNOWLEDGMENTS

This effort was supported (in part) by the U.S. Navy Bureau of Medicine and Surgery and by the intramural research program of the National Institute of Diabetes Digestive and Kidney Diseases Z01-DK062008 (RBM).

REFERENCES

1. Meier-Kriesche HU, Schold JD, Srinivas TR, Kaplan B: Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era, Am J Transplant 2004, 4:378-383
2. Racusen L: Chronic transplant glomerulopathy: need for further assessment, Clin J Am Soc Nephrol 2007, 2:1108-1109
3. Mauiyyedi S, Pelle PD, Saidman S, Collins AB, Pascual M, Tolkoff-Rubin NE, Williams WW, Cosimi AA, Schneeberger EE, Colvin RB: Chronic humoral rejection: identification of antibody-mediated chronic renal allograft rejection by C4d deposits in peritubular capillaries, J Am Soc Nephrol 2001, 12:574-582
4. Regele H, Bohmig GA, Habicht A, Gollowitzer D, Schillinger M, Rockenschaub S, Watschinger B, Kerjaschki D, Exner M: Capillary deposition of complement split product C4d in renal allografts is associated with basement membrane injury in peritubular and glomerular capillaries: a contribution of humoral immunity to chronic allograft rejection, J Am Soc Nephrol 2002, 13:2371-2380
5. Sis B, Campbell PM, Mueller T, Hunter C, Cockfield SM, Cruz J, Meng C, Wishart D, Solez K, Halloran PF: Transplant glomerulopathy, late antibody-mediated rejection and the ABCD tetrad in kidney allograft biopsies for cause, Am J Transplant 2007, 7:1743-1752
6. Akalin E, Dinavahi R, Dikman S, de Boccardo G, Friedlander R, Schroppel B, Sehgal V, Bromberg JS, Heeger P, Murphy B: Transplant glomerulopathy may occur in the absence of donor-specific antibody and C4d staining, Clin J Am Soc Nephrol 2007, 2:1261-1267
7. Al Aly Z, Yalamanchili P, Cortese C, Salinas-Madrigal L, Bastani B: C4d peritubular capillary staining in chronic allograft nephropathy and transplant glomerulopathy: an uncommon finding, Transpl Int 2005, 18:800-805
8. Gloor JM, Sethi S, Stegall MD, Park WD, Moore SB, DeGoey S, Griffin MD, Larson TS, Cosio FG: Transplant glomerulopathy: subclinical incidence and association with alloantibody, Am J Transplant 2007, 7:2124-2132
9. Cosio FG, Gloor JM, Sethi S, Stegall MD: Transplant glomerulopathy, Am J Transplant 2008, 8:492-496
10. Akalin E, Dikman S, Murphy B, Bromberg JS, Hancock WW: Glomerular infiltration by CXCR3+ ICOS+ activated T cells in chronic allograft nephropathy with transplant glomerulopathy, Am J Transplant 2003, 3:1116-1120
11. Hoffmann SC, Hale DA, Kleiner DE, Mannon RB, Kampen RL, Jacobson LM, Cendales LC, Swanson SJ, Becker BN, Kirk AD: Functionally significant renal allograft rejection is defined by transcriptional criteria, Am J Transplant 2005, 5:573-581
12. Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, Croker BP, Demetris AJ, Drachenberg CB, Fogo AB, Furness P, Gaber LW, Gibson IW, Glotz D, Goldberg JC, Grande J, Halloran PF, Hansen HE, Hartley B, Hayry PJ, Hill CM, Hoffman EO, Hunsicker LG, Lindblad AS, MARCUSSEN N, MIHATSCH MJ, NADASDY T, NICKERSON P, OLSEN TS, PAPADIMITRIOU JC, RANDHAWA PS, C RAYNER D, ROBERTS I, ROSE S, RUSH D, SALINAS-MADRIGAL L, SALOMON DR, SUND S, TASKINEN E, TRPKOV K, Yamaguchi Y: The Banff 97 working classification of renal allograft pathology, Kidney Int 1999, 55:713-723
13. Solez K, Colvin RB, Racusen LC, Sis B, Halloran PF, Birk PE, Campbell PM, Cascalho M, Collins AB, Demetris AJ, Drachenberg CB, Gibson IW, Grimm PC, Haas M, Lerut E, Liapis H, Mannon RB, Marcus PB, Mengel M, Mihatsch MJ, Nankivell BJ, Nickeleit V, Papadimitriou JC, Platt JL, Randhawa P, Roberts I, Salinas-Madriga L, Salomon DR, Seron D, Sheaff M, Weening JJ: Banff '05

Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'), Am J Transplant 2007, 7:518-526
14. Solez K, Colvin RB, Racusen LC, Haas M, Sis B, Mengel M, Halloran PF, Baldwin W, Banfi G, Collins AB, Cosio F, David DS, Drachenberg C, Einecke G, Fogo AB, Gibson IW, Glotz D, Iskandar SS, Kraus E, Lerut E, Mannon RB, Mihatsch M, Nankivell BJ, Nickeleit V, Papadimitriou JC, Randhawa P, Regele H, Renaudin K, Roberts I, Seron D, Smith RN, Valente M: Banff 07 classification of renal allograft pathology: updates and future directions, Am J Transplant 2008, 8:753-760
15. Hoffmann SC, Kampen RL, Amur S, Sharaf MA, Kleiner DE, Hunter K, John Swanson S, Hale DA, Mannon RB, Blair PJ, Kirk AD: Molecular and immunohistochemical characterization of the onset and resolution of human renal allograft ischemia-reperfusion injury, Transplantation 2002, 74:916-923
16. Hoffmann SC, Pearl JP, Blair PJ, Kirk AD: Immune profiling: molecular monitoring in renal transplantation, Front Biosci 2003, 8:e444-462
17. Kohavi R: A study of Cross-Validation and Bootstrap for Accuracy Estimation and Model Selection. Edited by Montreal, Canada, 1995, p.
18. Cosio FG, Grande JP, Wadei H, Larson TS, Griffin MD, Stegall MD: Predicting subsequent decline in kidney allograft function from early surveillance biopsies, Am J Transplant 2005, 5:2464-2472
19. Jensen FV: An introduction to Bayesian networks. Edited by New York, Springer, 1996, p
20. Grunkemeier GL, Payne N: Bayesian analysis: a new statistical paradigm for new technology, Ann Thorac Surg 2002, 74:1901-1908
21. Maskery SM, Hu H, Hooke J, Shriver CD, Liebman MN: A Bayesian derived network of breast pathology co-occurrence, J Biomed Inform 2008, 41:242-250
22. Montgomery SP, Swiecki CW, Shriver CD: The evaluation of casualties from Operation Iraqi Freedom on return to the continental United States from March to June 2003, J Am Coll Surg 2005, 201:7-12; discussion 12-13
23. Diamond GA, Forrester JS: Analysis of probability as an aid in the clinical diagnosis of coronary-artery disease, N Engl J Med 1979, 300:1350-1358
24. Staniloff HM, Diamond GA, Freeman MR, Berman DS, Forrester JS: Simplified application of Bayesian analysis to multiple cardiologic tests, Clin Cardiol 1982, 5:630-636
25. Cornell LD, Smith RN, Colvin RB: Kidney transplantation: mechanisms of rejection and acceptance, Annu Rev Pathol 2008, 3:189-220
26. Kamoun M: Cellular and molecular parameters in human renal allograft rejection, Clin Biochem 2001, 34:29-34
27. Kouwenhoven EA, de Bruin RW, Heemann UW, Marquet RL, JN IJ: Transplantation of a single kidney per se does not lead to late graft dysfunction, Transpl Int 2001, 14:38-43
28. Rodder S, Scherer A, Raulf F, Berthier CC, Hertig A, Couzi L, Durrbach A, Rondeau E, Marti HP: Renal allografts with IF/TA display distinct expression profiles of metzincins and related genes, Am J Transplant 2009, 9:517-526
29. Mengel M, Bock O, Priess M, Haller H, Kreipe H, Gwinner W: Expression of pro- and antifibrotic genes in protocol biopsies from renal allografts with interstitial fibrosis and tubular atrophy, Clin Nephrol 2008, 69:408-416
30. Homs S, Mansour H, Desvaux D, Diet C, Hazan M, Buchler M, Lebranchu Y, Buob D, Badoual C, Matignon M, Audard V, Lang P, Grimbert P: Predominant Th1 and cytotoxic phenotype in biopsies from renal transplant recipients with transplant glomerulopathy, Am J Transplant 2009, 9:1230-1236
31. Berthier C, Marti HP: Metzincins, including matrix metalloproteinases and meprin, in kidney transplantation, Swiss Med Wkly 2007, 137 Suppl 155:109S-114S
32. Palermo A, Mule G, Vadala A, Vaccaro F, Guarneri M, Arsena R, Briolotta C, Cerasola G, Cottone S: Relationship of transforming growth factor-beta(1) with tumour necrosis factor-alpha and endothelial activation in patients with stable renal transplantation, Nephrology (Carlton) 2008, 13:164-170

33. Meng L, Wu Z, Wang Y, Lassman C, Busuttil RW, Zhai Y, Kupiec-Weglinski JW: Differential impact of CD154 costimulation blockade on alloreactive effector and regulatory T cells in murine renal transplant recipients, Transplantation 2008, 85:1332-1338
34. Amirzargar M, Yavangi M, Basiri A, Moghadam SH, Khosravi F, Solgi G, Gholiaf M, Khoshkho F, Dadaras F, Mahmmodi M, Ansaripour B, Amirzargar A, Nikbin B: Genetic association of interleukin-4, interleukin-10, and transforming growth factor-beta gene polymorphism with allograft function in renal transplant patients, Transplant Proc 2007, 39:954-957
35. van den Boogaardt DE, van Miert PP, de Vaal YJ, de Fijter JW, Claas FH, Roelen DL: The ratio of interferon-gamma and interleukin-10 producing donor-specific cells as an in vitro monitoring tool for renal transplant patients, Transplantation 2006, 82:844-848
36. Kist-van Holthe JE, Gasser M, Womer K, Najafian N, Dong V, Samsonov DV, Geehan CS, Chandraker A, Sayegh MH, Waaga AM: Regulatory functions of alloreactive Th2 clones in human renal transplant recipients, Kidney Int 2002, 62:627-631
37. Sun Q, Liu ZH, Ji S, Chen J, Tang Z, Zeng C, Zheng C, Li LS: Late and early C4d-positive acute rejection: different clinico-histopathological subentities in renal transplantation, Kidney Int 2006, 70:377-383
38. Fotheringham J, Angel CA, McKane W: Transplant Glomerulopathy: Morphology, Associations and Mechanism, Nephron Clin Pract 2009, 113:c1-c7
39. Sarwal M, Chua MS, Kambham N, Hsieh SC, Satterwhite T, Masek M, Salvatierra O, Jr.: Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling, N Engl J Med 2003, 349:125-138
40. Muthukumar T, Dadhania D, Ding R, Snopkowski C, Naqvi R, Lee JB, Hartono C, Li B, Sharma VK, Seshan SV, Kapur S, Hancock WW, Schwartz JE, Suthanthiran M: Messenger RNA for FOXP3 in the urine of renal-allograft recipients, N Engl J Med 2005, 353:2342-2351
41. Lachenbruch PA, Rosenberg AS, Bonvini E, Cavaille-Coll MW, Colvin RB: Biomarkers and surrogate endpoints in renal transplantation: present status and considerations for clinical trial design, Am J Transplant 2004, 4:451-457
42. Allison M: Is personalized medicine finally arriving?, Nat Biotechnol 2008, 26:509-517
43. Molitoris BA, Melnikov VY, Okusa MD, Himmelfarb J: Technology Insight: biomarker development in acute kidney injury--what can we anticipate?, Nat Clin Pract Nephrol 2008, 4:154-165
44. Burckart GJ, Amur S, Goodsaid FM, Lesko LJ, Frueh FW, Huang SM, Cavaille-Coll MW: Qualification of biomarkers for drug development in organ transplantation, Am J Transplant 2008, 8:267-270
45. Mannon RB, Kirk AD: Beyond histology: novel tools to diagnose allograft dysfunction, Clin J Am Soc Nephrol 2006, 1:358-366
46. Nankivell BJ, Chapman JR: Chronic allograft nephropathy: current concepts and future directions, Transplantation 2006, 81:643-654

FIGURES

Figure 1. Transcriptional profile comparing stable function and transplant glomerulopathy allografts (GP1 and GP2). Transcript expression levels that were statistically different between SF (open bars) and TG (closed bars) allografts are shown ($p<0.05$). TG allografts showed significantly greater expression of transcripts related to **A)** T-cell activation and effector function, **B)** costimulatory molecules, **C)** chemotaxis, **D)** inflammatory cytokines and endothelial activation, **E)** epithelial-mesenchymal transformation, **F)** cytoskeleton structure, and **G)** growth factors and regulators of tissue remodeling when compared with SF allografts. Results are mean *n*-fold expression relative to normal, non-transplanted kidneys and depicted on a logarithmic scale. Error bars represent standard error of the mean (SEM).

Figure 2. Bayesian transcript network (GP1) and relationship to allograft pathology. **A)** The Bayesian transcript network structure of GP1 as established by the iterative modeling methods. The relative (*n*-fold) expression is represented for selected transcripts in three equal-area bins with associated probability distributions (blue bar) as predicted by the Bayesian model. In this model, ICAM-1, IL-10, CCL3, and CD86 were critically related to the allograft pathology variable 'Dx' (dashed box) as indicated by their adjacent location in the network. Multiple additional cytokine, chemokine, and costimulatory transcripts were also related to allograft pathology but not closely as indicated by their distance from the Dx variable. Transcripts not related to allograft pathology are outside of the network (BCL2, BAX, SKI, CSF1). **B)** With a SF allograft (set evidence is indicated by a black bar), the expression of multiple transcripts are decreased within the network, where decreased expression is represented in green and increased expression in red. The degree of shading represents the strength of the transcript relationship to the outcome, where darker is a stronger and lighter a weaker relationship. **C)** In an allograft with TG (black bar), the expression of the gene transcripts are increased in the Bayesian network.

Figure 3. Bayesian network probability analysis of allograft pathology based on transcript expression. **A)** With increased expression of ICAM-1 (≥1.84 fold), IL-10 (≥16.9 fold), and CCL3 (≥3.15 fold) (black bars) the probability of a TG allograft (dashed box) increases to 99.67%. **B)** With increased expression (>8.89 fold) of the costimulatory molecule CD86 (black bar), multiple related transcripts are also increased. For example, with increased expression of CD86 the probability of increased expression (>28.5 fold) of CD40L is 81.12%. In addition, the probability of a TG allograft (dashed box) also increased to 80.61%. Black bars indicate set evidence while blue bars indicate probability distributions within each graph.

Figure 4. Bayesian transcript network (GP2) and relationship to allograft pathology. **A)** In this model, VCAM-1, MMP-9, MMP-7, and LAMC2 are critically related to the allograft pathology (dashed box). In addition, the Banff C4d grade was included in this dataset and is also critically related to allograft pathology. The solid box indicates porting of network shown in panels B and C. **B)** With a Banff C4d grade of 3 (black bar), the probability of a TG allograft (dashed box) is 81.25%. **C)** However, with increased expression of VCAM-1 (≥1.96 fold), MMP-9 (≥5.34 fold), MMP-7 (≥2.77 fold), and LAMC2 (>2.19 fold) (black bars) the probability of a TG allograft (dashed box) increases to 99.67%. **D)** Bayesian prediction of allograft pathology based on C4d deposition. **E)** Bayesian prediction of allograft pathology based on combinations of LAMC2 and MMP7 expression levels. In panels D and E, "Probability of Case" reflects the occurrence rate of specified combination within the dataset. Low (green) to high (red) values are color coded.

TABLES

Table 1: Gene transcript targets

Gene Panel 1		Gene Panel 2	
BAX	EDN1	ACTA2	MMP9
BCL2	FASLG	ACTN4	NPHS1
C3	FOXP3	AFAP	NPHS2
CCL2	GATA3	AGRN	PDGFB
CCL3	GNLY	ANGPT2	S100A4
CCL5	GREM1	BMP7	SERPINE1
CCR1	GZMB	CD2AP	SERPINH1
CCR5	HLA-B1/3	CDH1	SMAD3
CD28	ICAM1	CDH3	SMAD7
CD3E	ICOS	COL1A1	SPARC
CD4	IFNG	COL3A1	SPP1
CD40	IL10	COL4A2	TGFB1
CD40LG	IL6	CTGF	THBS1
CD80	IL8	CTNNB1	TIMP1
CD86	MS4A1	FAT	TIMP2
CSF1	PDCD1	FGF2	TIMP3
CTLA4	PRF1	FN1	TIMP4
CX3CL1	SKI	GREM1	TJP1
CX3CR1	TBX21	HSPG2	TNC
CXCL10	TNF	IGF1	VCAM1
CXCL11		LAMC2	VEGF
CXCL9		MMP2	VIM
CXCR3		MMP7	

Table 2: Patient characteristics

Variable	SF	TG	p-value
Number of patients	19	18	
Recipient age (years)	38 ±14	42 ±14	NS[1]
Pre-transplant PRA peak	1.1 ±3.0	5.2 ±11.8	NS[1]
Donor age (years)	34 ±10	41 ±13	NS[1]
Donor type (%)			NS[2]
Living related	5 (26)	9 (50)	
Living unrelated	9 (48)	4 (22)	
Deceased	5 (26)	5 (28)	
HLA mismatch	3.6 ±1.7	3.4 ±1.5	NS[1]
Post-transplant DSA (%)	1 (5)	10 (56)	0.001[3]
First transplant (%)	18 (95)	15 (83)	NS[3]
Death-censored graft loss(%)	0	4 (22)	0.021[3]
Death with functioning graft (%)	0	3 (8)	NS[3]
Follow-up months	65.3 ±23.0	70.3 ±19.1	NS[1]

[1]Student t-test; [2]Chi square; [3]Fisher exact test

Table 3: Biopsy characteristics

	SF	**TG**	**p-value**
Number of biopsies	32	20	
Time from transplant (months)	23.31 ±19.3	32.7 ±16.3	NS[1]
Creatinine (at time of biopsy)	1.3 ±0.3	2.5 ±1.1	<0.001[1]
Biopsy for cause (%)	2 (6)	9 (45)	0.001[2]
Minimal changes	2	0	
Borderline rejection	0	2	
Acute humoral rejection	0	1	
Clinical toxicity	0	1	
Recurrent MPGN	0	2	
IF/TA	0	3	
C4d grade	0.6 ±0.9	1.6 ±1.1	0.013[1]
CG grade	0	2.7 ±0.5	<0.001[1]
IF/TA grade	0	1.4 ±1.2	<0.001[1]

[1]*Student t-test;* [2]*Chi squared*

Table 4: Fisher exact test of Bayesian associations in GP1

Association Pairs		**p-value**
Dx	ICAM1	<0.001
Dx	IL10	<0.001
Dx	CCL3	<0.001
CCL3	CD86	<0.001
CD86	CCL2	0.010
CD86	CXCL11	<0.001
CD86	CD40LG	<0.001
CD40LG	IFNG	0.004
CD40LG	CD28	<0.001
CXCL11	CD80	<0.001
CD80	CXCL10	0.001
CD80	GNLY	<0.001
GNLY	PRF1	0.001

Table 5: Fisher exact test of Bayesian associations in GP2

Association Pairs		**p-value**
Dx	VCAM1	<0.001
Dx	MMP9	<0.001
Dx	Banff.C4d	0.001
Dx	MMP7	<0.001
Dx	LAMC2	<0.001

Table 6: Sequential Biopsy Validation by Patient

Patient Number	Days Post Tx	Dx	Probability of SF	Probability of TG
002-003	1158	SF	0.038	0.962
	1830	SF	0.977	0.023
002-014	170	SF	0.153	0.847
	338	SF	0.024	0.976
002-017	177	SF	0.985	0.015
	1094	SF	0.985	0.015
	2088	SF	0.999	0.001
002-018	366	SF	0.958	0.042
	1099	SF	0.997	0.003
002-021	1092	TG	0.119	0.881
	1281	TG	0.002	0.998
002-023	1146	SF	0.646	0.354
	1840	SF	0.961	0.039
002-024	381	SF	0.971	0.029
	1099	SF	1.000	0.000
	1721	SF	0.791	0.209
002-031	194	SF	0.998	0.002
	368	SF	0.726	0.274
002-033	1287	TG	0.013	0.987
	1078	TG	0.003	0.997
002-035	198	SF	0.999	0.001
	363	SF	0.969	0.031
002-044	186	SF	0.810	0.190
	371	SF	0.999	0.001
002-047	177	SF	0.991	0.009
	359	SF	0.997	0.003
002-048	168	SF	0.998	0.002
	421	SF	0.980	0.020

Tx, transplant; Dx, diagnosis as defined in Methods; SF, stable function; TG, transplant glomerulopathy; probability greater than 0.500 was considered a positive test.

Figure 1

Bayesian Modeling of TG Gene Expression

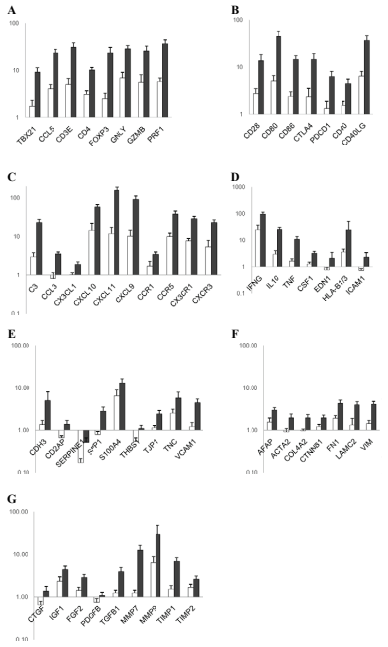


Figure 2

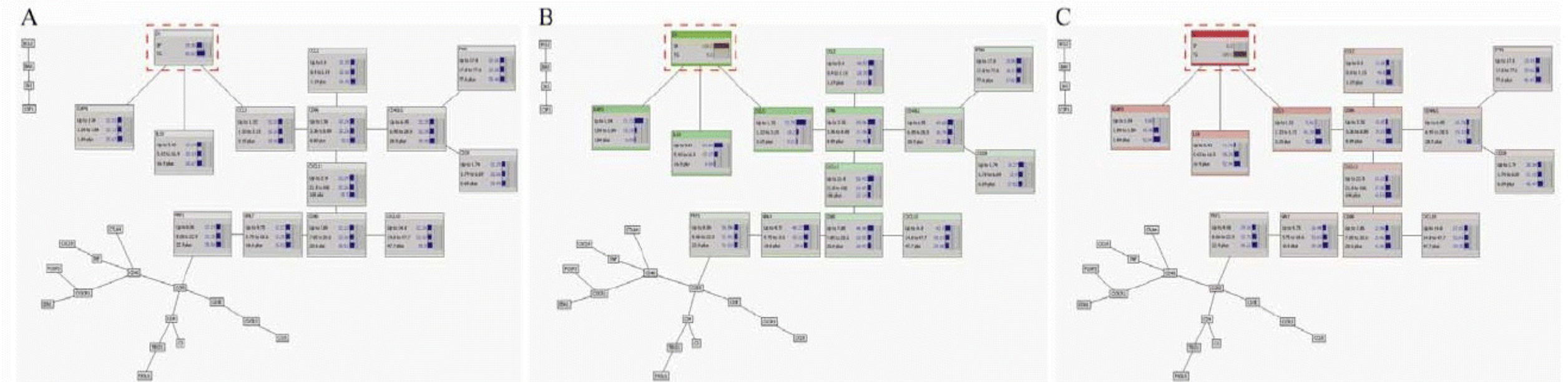


Figure 3

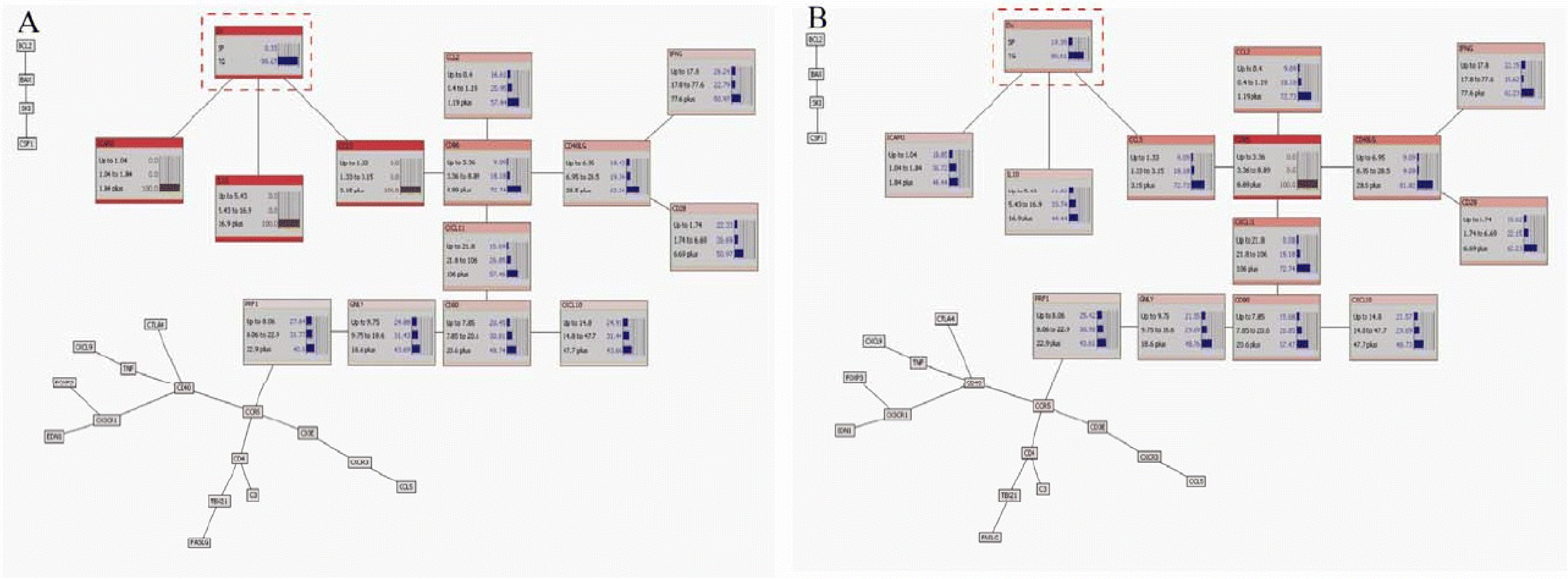
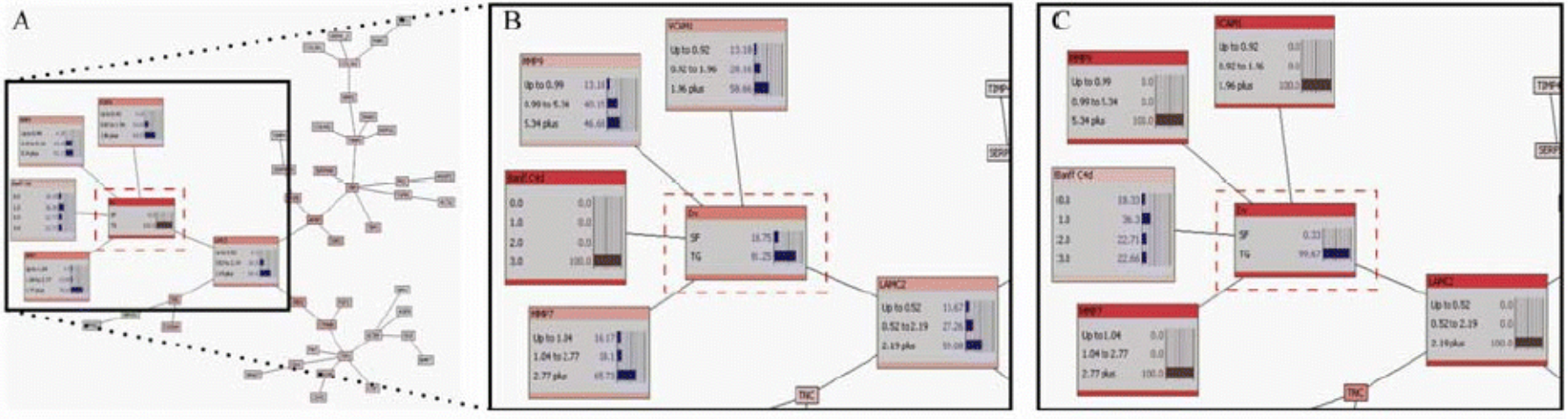

A
B
BCL2
BAX
SKI
CSF1
TNF
CCR5
CD4
C3
TBX21
FASLG
CCL5
EDN1
CTLA4
FOXP3

Figure 4



D

Banff C4d grade	Probability of Allograft Pathology		Probability of Case (%)
	SF (%)	TG (%)	
0.0	83.9	16.1	44.7
1.0	41.9	58.1	24.8
2.0	53.6	46.4	19.4
3.0	18.8	81.2	11.1

E

Transcript Expression		Probability of Allograft Pathology		Probability of Case (%)
LAMC2	MMP7	SF (%)	TG (%)	
<0.52	<1.04	99.1	0.9	16.0
0.52 to 2.19	<1.04	91.1	8.9	10.2
≥2.19	<1.04	63.6	36.4	6.6
<0.52	1.04 to 2.77	98.3	1.7	13.3
0.52 to 2.19	1.04 to 2.77	84.9	15.1	9.0
≥2.19	1.04 to 2.77	48.9	51.1	7.0
<0.52	≥2.77	73.5	26.5	5.1
0.52 to 2.19	≥2.77	21.1	78.9	10.3
≥2.19	≥2.77	4.4	95.6	22.5